Decision on manuscript AEM01306-08 Version 1.txt

From: marylynn.yates@ucr.edu
Sent: Tuesday, September 02, 2008 1:04 PM
To: Harwood, Valerie
Subject: Decision on manuscript AEM01306-08 Version 1

Dr. Valerie Harwood
University of South Florida
Dept. of Biology
4202 East Fowler Ave.
Tampa, FL 33620-5550
United States

Re: Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay (AEM01306-08 Version 1)

Dear Dr. Harwood:

Comments on your revised manuscript have been received from three members of the editorial board or ad hoc reviewers. The reviewers expressed a number of concerns about the manuscript. These include questions regarding the specificity of the markers for chickens (as described by Reviewer 1), the lack of some controls (as described by Reviewer 3), and the lack of sufficient data to "validate" the markers for other applications (See comments of Reviewers 1 and 2). In addition, it was felt that the presentation of the material was inadequate, and in some cases inappropriate, for a scientific journal. For these reasons, and the reasons in the attached reviews, I am unable to accept your manuscript for publication. The reviewer comments are attached and I believe that they will be helpful to you. Your interest to publish in Applied and Environmental Microbiology is very much appreciated.

Sincerely,
Marylynn V. Yates
Editor, Applied and Environmental Microbiology (AEM)

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REVIEWER 1:
Comments:
There are three issues with respect to the specificity and general applicability of the markers that are of concern. 1. Litter is commonly the waste produced by broiler farms with birds running around on bedding, but liquid waste is commonly generated by egg producing farms with battery-fed hens. How could the marker developed here detect litter but not liquid chicken waste? This might not be an issue in this particular watershed, but certainly could be elsewhere depending on what kind of agriculture is being undertaken. 2. The amplification of a goose and a duck sample with the 'litter-specific' primers suggests that avian species in general may be detected. 3. The widespread applicability of the markers needs to be tested with samples from outside the constrained study area. Based on these considerations, how can the authors conclude with confidence that a given water sample was not impacted by broiler chickens, layer chickens, migratory birds, or resident birds? Are the authors confident that these markers would be useful for investigators working in watersheds that have these potential multiple fecal sources?
There are numerous minor spelling, punctuation, inconsistencies in units of measure, and formatting errors in the text that need to be corrected. The location of commercial suppliers needs to be provided. The citation list needs to be carefully

reviewed for errors and edited to fit AEM format.

Running title: poultry
l. 72. Not sure if it is necessary or advisable to mention the legality of the situation.
l. 77 and thereafter. Change British units of volume and length to SI.
l. 88. Beef pats, rather than scats.
l. 115. ..on ice..
l. 142 .. E. coli genus-specific..
l. 186. plate read?
l. 210. the authors will have to present the results of the T-RFLP in figure or table. How many bands, what molecular weight, etc.
l. 216. You mean the size of the plasmid insert in each clone was determined to see if it corresponded to the restriction fragment of interest?
l. 251. a line of evidence for fecal contamination, not of human health risk.

REVIEWER 2:
Comments:
The subject manuscript describes a research to identify and validate a poultry-specific marker for use in fecal source tracking. Putative markers were identified through t-RFLP, sequenced, and primers developed for fragment detection by PCR. Sensitivity and specificity of the PCR markers were measured against fecal pools in the local setting (Oklahoma). Field validation was attempted by applying the candidate PCR assay to water samples in which poultry contamination was anticipated.

This reviewer takes no issue with the approach used to develop the host-associated marker nor the validation of the assay through sensitivity and specificity testing. The procedures followed in this regard represent the state of this rapidly evolving science. On the other hand, the information presented as field validation was, in fact, a field application and provided little information to support the utility of the marker assay for field application. The efforts described represent an uncontrolled trial from which no conclusions regarding the utility of the assay can be extracted. This critique does not necessarily mean that the authors' conclusions regarding sources of fecal contamination in the study area are unfounded, rather that the efforts did not support the main thrust of this manuscript as regards to assay development and validation. See main comments for more specifics.

This submission is stylistically weak. The authors use biased language, phrases frequently are redundant, important information sometimes is lacking, and presentation of information sometimes lacks sufficient organization. In sum, this reviewer feels that the authors provided an insufficiently finished product to the journal for consideration. See major comments for examples of stylistic flaws in the manuscript.

Major comments:
1. The report is not sufficiently finished. These are not infrequent, minor style issues but are pervasive and make the manuscript exceedingly difficult to understand (detailed in minor comments). The references are not stylistically consistent.
2. Frequently missing information: What cultivation media were used to grow the indicator bacteria (line 117); how long were samples in transit before they were received and processed within 12 hours (line 131); where is the accession information for the clones (line 141 or 197); how were the products sequenced (before line 149); what constitutes weak amplification in a nested qPCR (line 229; assumed qPCR because of footer in table 4); six putative markers were identified with assays developed (asterisk in Table 1), yet data for only 4 are presented (tables 2 and 3); what were the optimization steps used in development of the qPCR assay (line 235)
3. Quantitative reports of marker concentrations are compromised by the lack of a recovery efficiency control in these samples. Recovery efficiency can vary wildly.

4. The conclusion of the abstract, "potential for determining fecal source allocations for TMDL programs" is not supported by the manuscript. TMDL programs almost universally rely upon E. coli or fecal coliforms to indicate the total amount of fecal contamination. It follows that a fecal source allocation for TMDLs will not be possible without a strong relationship between E. coli density and marker concentration in poultry litter. No such relationship was demonstrated in this research (Figure 3). The authors correctly report this fact in lines 289 and 290, contradicting the statement in the abstract. More samples will not alleviate this condition unless marker concentration and fecal indicator density in reference material are shown to be related with a slope of 1:1 as concentration increases (counter to what is shown in Figure 3, slope 0.6:2.5 for enterococci and weak relationship for E. coli).

5. The analysis of relationships between E. coli or enterococci density and putative poultry marker concentration in water is incomplete. In any given water sample, fecal contamination from any number of sources may be present. Thus, any validation for a relationship between poultry marker and fecal indicator must take into account the expected level of poultry contamination. Importantly, the ratio of marker to indicator would be relatively low for water with lesser poultry-origin contamination (bulk river water, especially upstream from poultry-amended fields), and relatively high with concentrated poultry contamination (as expected in runoff from litter-amended fields). Lumping all water samples, without regard to the expected level of poultry-origin contamination, and looking for a direct correlation is not particularly informative and does not constitute validation. Despite not presenting land-use information, the authors present (line 285) the suggestion that land use and level of contamination by poultry litter are correlated.

6. Correlation of poultry marker with fecal indicators (line 252) does not provide any evidence of human health risk. The relationship of fecal indicators with human health risk was developed at sites contaminated primarily with human waste (Dufour's publications, 1984 and 1986). This relationship is not expected to be the same for water contaminated with feces from nonhuman sources.

Minor comments:

39 - biased language

40 - pollution contaminating is redundant

44 - space missing between text and reference

46-49 - this description of the state of regulatory fecal-indicator bacteria in the United States likely will confuse both native and international readers. While recognizing that the focus area is Oklahoma in the United States, generalize the content to be relevant to an international audience. Suggest three sentences that summarize the state of regulations at the local, national, and international scale. You already have state and national to work from, for international consider WHO documentation for E. coli density criteria in bathing waters.

50-53 - run on sentence, awkward and difficult to understand

53-56 - run on sentence

58 - MST markers typically are not proposed as alternatives to monitoring fecal-indicator bacteria. One exception is general Bacteroidales in the USEPA (Wade) epidemiological study. MST markers more commonly are proposed to augment information about total contamination levels (fecal indicator bacteria densities) with information about sources (presence or relative abundance of fecal contamination from a targeted source).

64 - The compound noun "Host marker specific targets" is complex to the point of being nearly nonsensical.

67 - Misspelling, "Bacteroides"

69 - Two non-Bacteroides markers with purported specificity to poultry (CB-R2-42, CP1-25) were proposed in the cited article.

72-73 - Relevance of this statement to the content of the article. This information is presented already, appropriately, in the acknowledgements.

75 - This section really needs to be split into paragraphs. Recommend paragraph breaks at line 80, 84, 101 to enhance readability.

80 - How many fields were sampled? Where were they?

81 - Predetermined grid pattern is redundant.

82 - The 0 to 2-inch layer

83 - Did vegetation, feathers and rocks make it through the 2 mm sieve?

87 - Two independent duplicate samples is redundant.
88-89 - The summation of 200 cattle pats is not needed after the prior description.
90 - Independent duplicates is redundant 95, 96 - 2 duplicate samples is redundant
97 - Comma needed after each)
102 - A churn splitter is not a sample collection method
108 - Filtration is only part of the processing samples underwent. Suggest "filtered to recover total DNA and aliquoted for most-probable-numbers analysis of fecal-indicator bacteria." Were there other processes as well?
114 - All samples; it seems likely that feces, soil, and litter samples also were shipped to the laboratory for filtration.
117-121 - This method is very imprecise.
125 - Suggest inserting "further", as "was further purified" for clarity.
130 - Terminology. Likely the authors meant re-dissolved (if traditional ethanol precipitation) or, in line 129, captured and washed (if spin filters were used).
132-3 - Were Supor filters frozen to facilitate shattering?
142 - Perhaps from lack of experience with the cited primers, this reviewer is at a loss to understand how the E. coli-specific primers are described as genus-specific as well as how a non Escherichia clone (T-RF 500.8) was derived from the PCR products.
144 - Extraction should be the noun, extract.
187 - Units. The qPCR standards are (oddly) in units of ng/uL in the materials and methods but in units of copies/uL in Figure 2.
190 - Since these are plasmid-based standards (line 187), and not based on cultivated Brevibacterium, then the assumption should be one insert copy per plasmid?. There appears to be little reason to extrapolate from copy number to genome number in the original sample. Question the use of the term "gene" for this DNA sequence.
195 - suggest substituting "and" for "were."
210 - The statement about "the two litter and two soil samples" makes it appear that those were the only samples in the study. This is counter to the presentation of sample collection from 10 poultry houses in line 75.
214-216 - Information belongs in Methods
227 - Suggest offsetting LA35 with commas for clarity.
229 - Were the assays developed accomplished by end-point PCR or by quantitative PCR? Table 4 footnote a says qPCR. If so, what constitutes a weak amplification?
235 - Optimization steps not listed in Methods.
251-254 - Run on sentence leads to ambiguity about whether "these organisms" (line 234) refers to regulatory fecal indicators or Brevibacterium.
254 - The citation to a MST review article is non-ideal to support the concept of fecal indicator bacteria regrowth in the environment.
272, 272 - Clarify whether the intent of source tracking is to address eutrophication, recreational use impairment, or both.
274 - Biased language. There is no sanitary criterion for soil that would justify the use of "contaminated."
278 - Format of reference citation (26)
279 - Suggest further differentiating this study from the cited study by specifying that the cited study used fresh chicken feces as starting material, whereas this study used "aged" material that is more likely to actually contaminate a water body.
279 to 283 - The comparison is not valid and should be removed or expanded upon. This study used fecal composites, whereas the cited study used samples from individual chickens. There is no evidence that the marker in this study is more broadly distributed in individual poultry than are the markers in the cited study.
301 - Ambiguous terminology, using separate to mean independent but the samples actually were composite samples (not from separate, as in from individual animals).
301 - Space missing between against and 10
302 to 304 - No land-use data presented to support the expectation of variable concentrations of biomarker. This issue needs to be addressed throughout the paper.
306 - Suggest sentence break " …concentration. The correlation was …" for clarity
307 - Suggest "… Brevibacterium spp. Based marker for microbial …"
308 - "… among the first … methods …"
308 - Though the marker is quantifiable, the evidence presented in this paper does not indicate that use of the marker is sufficient to quantifiably track poultry fecal sources in environmental waters, at least in the sense of 10% of E. coli or

nutrients in this water body came from poultry-derived fecal contamination.
347 - Question whether the last three authors on this paper all have the same name.
392 - Note Santo Domingo, not Domingo
Table 1. 20 profiles (5 subsamples*4 samples) were generated, yet only 19 appear in Table 1.
It would be nice to order the t-RF and PCR reactions consistently in Tables 1-3.
Figure 2, how many replicates? Dynamic range? Matrix effects?
Table 4 - the difference between a "detection" and a "quantification" is not sufficiently described.
Figure 3 - The X-axis should be labeled biomarker, not Brevibacterium, to reflect the actual measurement. Ibid Figure 4

REVIEWER 3:
Comments:
The aim of this study was to identify a poultry litter-specific biomarker, to determine its specificity against other fecal sources and define a 16S rRNA real-time PCR for quantifying the proposed biomarker in environmental samples. Authors defined properly the purpose of the study and used adequate procedures. However, control materials (samples) are missing in order to determine the feasibility of the approach to a real situation in the environment. The use of DNA standards for real-time PCR is necessary but they are just providing internal control for the molecular method but not for controlling the usefulness and the detection limits when applied on environmental samples.

Other comments:

Page 2 lines 30 -32. Please, consider to remove this sentence. It is not supported by the present study.

Page 4 lines 72 - 73. This sentence is not scientifically relevant. It is already indicated on acknowledgements. Please, remove it.

Page 5 line 78 - 82 (and over the rest of manuscript). As it is indicated on "AEM Instructions for authors", it is preferable to use the Système International d'Unités (SI). Please, follow instructions for authors.

Page 5 line 85. Please, consider to add "…. From groups of individuals (cattle, duck, swine and human sewage):" It could avoid citing Table 3 on text before than Table 1 and 2. Revise number of tables and their appearance order on the text.

Page 7 line 131. Volume of water samples is missing. It should be indicated to support detection limits of the performed analyses.

Page 7 line 142. Citation on text. Please, revise it all along the manuscript by following "AEM Instructions for authors".

Page 8 line 155. Bacteria should not be in italics. The term is not referring genus neither species. Please, revise it all along the manuscript (for instance, lines 171, 173, 213 and so on).

Page 9 line 167. Again, consider to revise numbering for tables.

Page 11 line 212. Again, consider to revise numbering for tables.

Page 11 lines 214 - 215. Please, move this sentence to M&M.

Page 11 lines 225. Was it nested PCR? If so, please, explain on M&M, it is not clearly indicated. For instance, qPCR (on M&M) is only reported to be used to amplify the 16S rRNA gene from Brevibacterium spp. DNA samples (lines 177 - 178). Moreover, what means Brevibacterium spp. DNA samples? Non-tergat environmental samples) or extracted DNA from a collection strains of Brevibacterium?
Page 12 line 238. Please, consider to add: "…. of extracted DNA when using clone plasmid DNA standards".

Page 12 lines 234 - 244. The detection limit of any new molecular methods for environmental use should be determined using DNA standards obtained similarly to DNA environmental samples (control materials). For instance, Brevibacterium spp. type strains culture suspensions prepared using a similar water matrix (and a serial of ten-fold dilutions for calibration) should be assayed following concentration and DNA extraction procedures used later for environmental samples, and next the defined qPCR assay. Such kind of internal control suspensions would provide the real detection limit of the new molecular when applied on environmental samples. Clone plasmid DNA standards are good control just for the qPCR analytical method. Have authors checked the proposed procedure with this kind of control samples?

Page 12 line 247. Figures 3 and 4 are not necessary to support the content of text. Both figures could be removed.

Page 12 lines 253 - 254. The evidence for regrowth of E. coli and other intestinal microorganisms is questioned by some researchers. Please, consider to remove the last sentence of the paragraph (….although there is ….into the environment (36)). It is not supported by the present work and is out of scope for the paper.

Page 13 line 256. "Validated" is not an appropriated term attending to the performed study. Please, consider the revision of this paragraph by checking ISO/TR 13843 (Water Quality - Guidance on validation of microbial methods) for the definition of validation and what it requests.

Page 14 line 298. Please, remove subheading by following "AEM Instructions for authors".

Page 22. Table 1. Please, avoid repetition of number of subsamples tested all along the columns. It could be solved by adding to the table heading "…had been applied. Number of subsamples containing T-RF of interst. n, number of subsamples tested". Then, remove this text on the table, put below Litter A (n=4), Litter B (n=5) and so on, and remove "n" values on each column by keeping without parenthesis the respective number of subsamples containing T-RF of interest. It would simplified the table.

Page 24. Table 3. Similarly to Table 1. Please, consider to chance Table heading to: "Specificity of the poultry litter biomarker nester PCR assay tested against fecal samples from within and outside the watershed. N, number of tested samples". Then, add a column (label, n) after the first column for the number of samples tested, and avoid the repetition of n on each column. Parenthesis and text on the table "Number of smaples tested" should be removed.

Page 26. Figure 2 is not necessary if it is commented on text.

Page 27. Table 4. The estimation of the corresponding number of cells/100 ml is suggested to be indicated. The reported concentration of biomarker could be moderate or low at point source in terms of cells. Bacteria which are alive or metabolically active could have easily 10E4 ribosomes (targets) by cell. To know the concentration of the proposed MST indicator at point source by using common units for most of microbial water indicators is convenient. It has been described that high concentration of MST indicators at point source are necessary otherwise they will be not feasible because they could not be measured after dilution and die-off on the environment.